

Effect of high-speed centrifugation on the sensitivity of irradiated McCoy cell culture for the isolation of *Chlamydia*

S. DAROUGAR, S. CUBITT, AND B. R. JONES

Virus Laboratory, Department of Clinical Ophthalmology, Institute of Ophthalmology, London

Studies by Weiss and Dressler (1960) and Gordon, Quan, and Trimmer (1960) have shown that centrifugation of isolates of trachoma or psittacosis agents as well as *Rickettsia prowazeki* onto cell monolayers will increase the sensitivity of cell culture several fold.

Using centrifugal force to facilitate the precipitation of infective particles onto irradiated McCoy cells, Gordon, Magruder, Quan, and Arm (1963) and Gordon, Dressler, and Quan (1967) developed a cell culture technique as an alternative to cultivation in the yolk sac of fertile eggs for the primary isolation of *Chlamydia*. This technique has been shown to be three to four times more sensitive than the isolation of *Chlamydia* in the yolk sac or the detection of inclusion bodies in smears (Gordon, Harper, Quan, Treharne, Dwyer, and Garland, 1969; Darougar, Treharne, Dwyer, Kinnison, and Jones, 1971). It has since been extensively used in our laboratory for the isolation of *Chlamydia* from the eye, genital tract, and rectum of patients in the United Kingdom (Gordon and others, 1969; Dunlop, Hare, Darougar, Jones, and Rice, 1969; Dunlop, Vaughan-Jackson, Darougar, and Jones, 1972) and from the eye in the cases of patients suffering from hyperendemic trachoma in Iran (Darougar and others, 1971). These techniques have now been successfully used for routine isolation of *Chlamydia* in other laboratories (Oriol, Reeve, Powis, Miller, and Nicol, 1972; Richmond, Hilton, and Clarke, 1972).

The force of centrifugation used in the original technique of culture in irradiated McCoy cells was $1,800 \times G$ for 1 hr at $18^{\circ}C$. The purpose of this study was to investigate the effect of higher temperatures during centrifugation, and higher forces of centrifugation, as well as to determine the optimum period of centrifugation for isolation and cultivation of *Chlamydia* in irradiated McCoy cells.

Material and methods

SOURCE OF SPECIMENS

Specimens used in this study were harvests of *Chlamydia* which had been isolated either in irradiated McCoy cell culture or egg culture, or clinical specimens which were collected from the eye or genital tract of patients in London who were suffering from infection of the eye and genital tract by TRIC agent. None came from men presenting with non-specific urethritis without eye disease.

METHODS OF COLLECTION OF SPECIMENS

Specimens from the eye, collected by swabbing different areas of the conjunctiva, were placed in plastic capsules containing 2SP transport medium with additional serum (Darougar, Jones, Kinnison, Vaughan-Jackson, and Dunlop, 1972). Specimens from the genital tract, collected by swabbing or scraping the urethra and swabbing or scraping the cervix, were placed in plastic capsules containing 2SP with serum (Dunlop, Vaughan-Jackson, and Darougar, 1972). All specimens were stored in a liquid nitrogen refrigerator for transportation to the laboratory and were stored there in a $-70^{\circ}C$. refrigerator until the time of inoculation.

CELL CULTURE TECHNIQUE

After irradiation, monolayers of McCoy cells were prepared in flat-bottomed tubes (bijou bottles, Sterilin Ltd., Richmond, Surrey) containing coverslips. Except for the temperature maintained during centrifugation and the time and force of centrifugation, the techniques used were those of Darougar and others (1971).

CENTRIFUGATION OF CELL CULTURE

The following types of centrifuges were used:

- MSE Super-Minor Bench Centrifuge with wind-shield for forces of $2,500 \times G$.
- MSE Refrigerated Major Centrifuge for forces of $1,800 \times G$.
- MSE High Speed 25 Centrifuge for forces up to $15,000 \times G$.
- Sorval Superspeed RC2-B Refrigerated Centrifuge for forces up to $15,000 \times G$.

TEMPERATURE AND FORCE OF CENTRIFUGATION

Experiments were carried out to investigate the following:

(1) *Effect of centrifugation at 35°C. in comparison with centrifugation at 18°C.*

Aliquots of a harvest of TRIC agent grown originally in cell culture or egg culture were each inoculated into four tubes containing cell monolayers. Of these four tubes, two were centrifuged at $1,800 \times G$ for 1 hr at 18°C, and the other two at $1,800 \times G$ for 1 hr at 35°C.

(2) *Effect of centrifugation in a bench centrifuge in comparison with centrifugation in a refrigerated centrifuge*

Aliquots of a harvest of TRIC agent grown originally in cell culture were each inoculated into four tubes containing cell monolayers. Of these four tubes, two were centrifuged in a refrigerated centrifuge at $1,800 \times G$ for 1 hr at 35°C., and the other two in an MSE Super Minor bench centrifuge with a windshield at $2,500 \times G$ for 1 hr at the maximum temperature of 35° to 38°C.

(3) *Effect of centrifugation at $15,000 \times G$ in comparison with centrifugation at $2,500 \times G$ and with no centrifugation*
Clinical specimens, or dilutions of clinical specimens, were each inoculated into six tubes containing cell monolayers. Of these six tubes, two were centrifuged at $15,000 \times G$ at 35°C for 1 hr, two at $2,500 \times G$ for 1 hr in a bench centrifuge with a maximum temperature of 35° to 38°C., and the last two were incubated without centrifugation.

(4) *Effect of centrifugation at $15,000 \times G$ in comparison with centrifugation at $10,000 \times G$*

Clinical specimens, or dilutions of clinical specimens, were each inoculated into four tubes containing cell monolayers. Of these four tubes, two were centrifuged at $15,000 \times G$ for 1 hr at 35°C. and the other two at $10,000 \times G$ for 1 hour at 35°C.

(5) *Effect of centrifugation at $15,000 \times G$ for times varying from 15 to 60 min.*

Clinical specimens, or dilutions of them, were each inoculated into eight tubes containing cell monolayers. All eight inoculated tubes were centrifuged at $15,000 \times G$ at 35°C. Two tubes were removed after 15, 30, 45, and 60 min. of centrifugation.

(6) *Effect of centrifugation at $1,800 \times G$ for times varying from 1 to 3 hrs*

Clinical specimens, or dilutions of them, were each

inoculated into six tubes containing cell monolayers. All six tubes were centrifuged at $1,800 \times G$ at 35°C. Two tubes were removed after 1, 2 and 3 hrs of centrifugation.

QUANTIFICATION OF INFECTIVITY IN CELL MONOLAYERS

After approximately 60 hrs' incubation, the inoculated monolayers were fixed and stained with Giemsa's stain (Darougar and others, 1971). Using a Zeiss RA microscope, Giemsa-stained coverslips were examined for the presence or absence of inclusions with a $10 \times$ objective, $2 \times$ optovar, and $10 \times$ eye piece using dark-field illumination. In addition, the number of inclusions in the whole area of each monolayer was recorded for comparison between techniques. Monolayers with degenerated cells or defective areas were excluded from the study.

Results

Effect of centrifugation at 35°C. in Comparison with centrifugation at 18°C.

Harvests of four TRIC isolates grown in cell culture, and three grown in fertile eggs were used in this study. The total number of inclusions formed after centrifugation of specimens at 35°C. in comparison with 18°C. is shown in Table I. The ratio of inclusions formed after centrifugation at 35°C. to inclusions formed at 18°C. ranged from 1.5 to 7.3 (mean 4.1 for all specimens). This difference is significant at the level of $P < 0.02$. Additionally, the inclusions formed after centrifugation at 35°C. were larger, more mature, and more easily detected than those formed after centrifugation at 18°C.

Effect of centrifugation in a bench centrifuge (at $2,500 \times G$ and temperatures of 35 to 38°C.) in comparison with centrifugation in a refrigerated centrifuge (at $1,800 \times G$ and a temperature of 35°C.)

Six specimens consisting of dilutions of harvests of two strains of TRIC agent isolated in cell culture were used in this study. The total number of inclusions formed after centrifugation of specimens in a bench centrifuge was slightly higher in comparison with

TABLE I *Number of inclusions obtained after centrifugation of TRIC isolates at 18° and 35°C.*

Isolate designation	Origin	Number of inclusions		Ratio of inclusion counts 35°C.: 18°C.
		18°C.	35°C.	
TRIC//IR/IOL-221/OT	Cell culture	4	29	7.3
TRIC//IR/IOL-243/OT		5	8	1.6
TRIC//GB/IOL-217/GCX		166	727	4.3
TRIC//GB/IOL-202/ON		6	30	5
TRIC//IR/IOL-231/OT	Egg culture	1,086	1,625	1.5
TRIC//IR/IOL-223/OT		7	47	6.7
TRIC//IR/IOL-225/OT		59	158	2.6
Mean ratio of inclusions				4.1 ^a

^a $P < 0.02$

the number of inclusions formed after centrifugation in a refrigerated centrifuge (Table II). This difference might be due to the slightly higher force of centrifugation employed in the bench centrifuge, but is not statistically significant.

TABLE II *Number of inclusions obtained after centrifugation of TRIC isolates in a bench centrifuge ($2,500 \times G$ at 35 to 38°C) in comparison with centrifugation in a refrigerated centrifuge ($1,800 \times G$ at 35°C.)*

Isolate designation	Number of inclusions	
	Bench centrifuge	Refrigerated centrifuge
TRIC//IR/IOL-237/OT	1,707	1,567
TRIC//IR/IOL-243/OT	494	443
TRIC//IR/IOL-237A/OT	253	174
TRIC//IR/IOL-243A/OT	56	53
TRIC//IR/IOL-237B/OT	12	5
TRIC//IR/IOL-243B/OT	4	1

Effect of centrifugation at $15,000 \times G$ in comparison with centrifugation at $2,500 \times G$ and no centrifugation at all

Dilutions of 47 clinical specimens (neat, $\frac{1}{8}$, and $\frac{1}{64}$) were used in this study. Of a total of 141 specimens inoculated, 57 were positive using the centrifugal force of $15,000 \times G$ in comparison to 46 positive using the force of $2,500 \times G$, and only three positive using no centrifugation at all (Table III). This difference in the rate of positivity between the two forces of centrifugation is statistically significant at the level of $P < 0.05$. As may be seen in Table III, the effect of the higher force of centrifugation is more evident with higher dilutions of clinical specimens when a lower number of infective particles were inoculated. Going along with the higher rate of positivity, the number of inclusions obtained in positive specimens after centrifugation at $15,000 \times G$ was higher with all specimens, including those of high infectivity that gave a similar high rate of positivity with either gravity force. The mean ratio of inclusions obtained in positive specimens after

TABLE III *Effect of centrifugation at $15,000 \times G$, $2,500 \times G$, or no centrifugation on isolation of Chlamydia from clinical specimens*

No. of clinical specimens*	Dilution	No. of positive cultures		
		$15,000 \times G$	$2,500 \times G$	No centrifugation
47	Neat	27	26	3
47	1/8	25	19	0
47	1/64	5	1	0
Total	141	57 ^b	46 ^b	3

*Sources of specimens: cervix 6; female urethra 9; male urethra 3; conjunctiva 29

^b $P < 0.05$

centrifugation at $15,000 \times G$ was 3.6 times higher than the number of inclusions obtained after centrifugation at $2,500 \times G$.

Effect of centrifugation at $15,000 \times G$ in comparison with centrifugation at $10,000 \times G$

Dilutions of eleven clinical specimens (neat, $\frac{1}{8}$, and $\frac{1}{64}$) were used in this study. Of a total of 33 specimens inoculated, eighteen were positive using the centrifugal force of $15,000 \times G$ in comparison to fifteen positive specimens obtained using the force of $10,000 \times G$ (Table IV). Although the difference in the rate of positivity between these two forces of centrifugation did not reach statistical significance, the mean ratio of inclusions formed after centrifugation of specimens at $15,000 \times G$ was 50 per cent. higher than the ratio of inclusions formed at $10,000 \times G$, and this difference is significant at the level of $P < 0.05$.

TABLE IV *Effect of centrifugation at $15,000 \times G$ and $10,000 \times G$ on isolation of Chlamydia from clinical specimens*

No. of clinical specimens	Dilution	No. of positive cultures	
		$15,000 \times G$	$10,000 \times G$
11	Neat	8	7
11	1/8	6	6
11	1/64	4	2
Total	33	18	15

Effect of centrifugation at $15,000 \times G$ for 15 to 60 min.

Seven clinical specimens were inoculated onto cell cultures and centrifuged at $15,000 \times G$ for the periods ranging from 15 to 60 min. In each case the number of inclusions formed increased with increasing time of centrifugation. The total number of inclusions from all clinical specimens after 1 hour of centrifugation was 38 and 107 per cent. higher than those obtained after 45 and 30 min. respectively (Table V).

TABLE V *Effect of centrifugation at $15,000 \times G$ for 15 to 60 min. on isolation of Chlamydia from clinical specimens*

Specimen code no.	Number of inclusions			
	15 min.	30 min.	45 min.	60 min.
10	417	1,269	1,872	2,612
12	142	370	689	847
13	52	82	93	143
14	3	25	29	36
15	28	36	43	59
16	36	38	42	72
17	31	59	192	294
Mean ratio of inclusions	1	2.8	4.2	5.8

Effect of centrifugation at $1,800 \times G$ for 1 to 3 hrs

Ten clinical specimens were inoculated onto cell cultures and were centrifuged at $1,800 \times G$ for 1 to 3 hrs. The number of inclusions obtained after 3 and 2 hrs centrifugation was only 16 and 18 per cent. respectively, higher than the percentage of inclusions obtained after 1 hr's centrifugation (Table VI). The difference in the yield of inclusions after 1 hr and 3 hrs' centrifugation is not statistically significant.

TABLE VI *Effect of centrifugation at $2,500 \times G$ for 1 to 3 hours on isolation of Chlamydia from clinical specimens*

Specimen code no.	No. of inclusions		
	1 hr	2 hrs	3 hrs
21	1,186	1,358	1,598
22	155	232	193
23	192	222	190
24	238	294	288
25	14	19	23
26	547	536	541
27	168	169	187
28	822	898	844
29	263	282	278
30	13	18	13
Mean ratio of inclusions	1	1.18	1.16

Discussion

The striking increase in sensitivity after centrifugation of clinical specimens (Table III) confirms the importance of centrifugation in primary isolation of *Chlamydia* in irradiated McCoy cell culture.

The force of centrifugation used in the original technique (Gordon and others, 1969) was $1,800 \times G$ for 1 hr at 18°C . The choice of 18°C . during the period of centrifugation appears to have been made as a compromise between thermal inactivation of *Chlamydia* and inhibition of host-parasite interaction (Weiss and Dressler, 1960). In this study the sensitivity of cell culture was increased significantly when specimens were centrifuged at 35°C . (Table I). The results of the studies by Jawetz and Hanna (1960), and the present work, indicate that the loss of infectivity of *Chlamydia* during a short period at 35°C . is not significant. Therefore, it may be concluded that the increase in the sensitivity of cell culture after centrifugation at 35°C . is due to the enhancement of host-parasite interaction, particularly the phagocytic activity of cells during the period of centrifugation.

The practicability of using a bench centrifuge simplifies the method of irradiated McCoy cell culture and facilitates the application of this technique to the study of chlamydial infection of the genital tract in field studies of hyperendemic trachoma in developing countries. The presence of a windshield in the bench centrifuge is an important factor in con-

trolling the temperature during centrifugation. In areas where the room temperature is high, the centrifuge lid could be lifted up slightly to allow a free circulation of air around the windshield, thus reducing the temperature inside the centrifuge. This technique is likely to remain of use in field laboratories or others not having suitable facilities for high speed centrifugation.

The choice of a lower centrifugal force of $1,800 \times G$ in the original technique of irradiated McCoy cell culture was probably made because of the lack of suitable tubes that can withstand a higher force of centrifugation, and on the assumption that the infective chlamydial particles are generally enveloped in cell debris or intact cells which are large enough to be deposited onto the cell monolayer using a low centrifugal force.

The use of Sterilin bijou bottles has enabled us to employ a higher centrifugal force of up to $15,000 \times G$ without breakage or loss of specimens. The investigation of the effect of different levels of centrifugal force and periods of centrifugation (Tables III, IV, and V) has indicated that centrifugation at $15,000 \times G$ for 1 hr at 35°C . is superior to any lesser force and shorter period of centrifugation.

The clinical specimens used in this study were prepared and inoculated as neat, $\frac{1}{2}$, and $\frac{1}{4}$ dilutions in order to mimic clinical specimens with different degrees of infectivity. It is interesting that, when the specimens contained a large number of infective particles, the lower centrifugal force of $2,500 \times G$ was sufficient to yield a rate of positivity of culture comparable to that obtained with the centrifugal force of $15,000 \times G$, but in the case of specimens containing few infective particles centrifugation at $15,000 \times G$ increased significantly the rate of positivity. Using low speed centrifugation ($2,500 \times G$ for 1 hr at 35 to 38°C .), the rate of isolation of *Chlamydia* from the urethra in the cases of men presenting in London with so called 'non-specific' urethritis (NSU) was 44 per cent. (Dunlop and others, 1972). Using the same technique, the rate of positivity from conjunctival material from patients suffering from hyperendemic trachoma in Iran was 28 per cent. (Darougar and others, 1971). The use of the higher force of $15,000 \times G$ may be expected to increase significantly the yield of positive results, especially from mild or more chronic phases of either of these chlamydial diseases when little viable material can be collected.

We are developing a technique for chlamydial isolation in irradiated McCoy cells using a micro-tube which will increase the through-put of a high-speed centrifuge several fold, and make it possible to use the higher centrifugal force as a routine.

Summary

Centrifugation of clinical specimens at $1,800 \times G$ at $18^\circ C$. for 1 hr increases significantly the sensitivity of irradiated McCoy cell culture for the primary isolation of *Chlamydia*. With a higher temperature of $35^\circ C$. during the period of centrifugation, the number of inclusions obtained was 4.1 times higher than the number of inclusions obtained after centrifugation at $18^\circ C$. The number of inclusions obtained after centrifugation of laboratory isolates in a bench centrifuge with a windshield at $2,500 \times G$ at 35° to $38^\circ C$. for 1 hr was slightly higher than the number of inclusions obtained after centrifugation of these specimens in a refrigerated centrifuge at $1,800 \times G$ at $35^\circ C$. for 1 hr.

This observation provided the basis for using a simple bench centrifuge for the routine isolation of *Chlamydia*. This technique remains useful for field laboratories and others not having facilities for high-speed centrifugation.

Centrifugation of clinical specimens having a low titre of infectivity at $15,000 \times G$ at $35^\circ C$. for 1 hr increased significantly the rate of positivity in irradiated McCoy cell culture in comparison with centrifugation at $2,500 \times G$ at $35^\circ C$. for 1 hr; it also increased significantly the number of inclusions obtained from clinical specimens in comparison with centrifugation at either $2,500$ or $10,000 \times G$ at $35^\circ C$. for 1 hr.

The application of a higher centrifugal force ($15,000 \times G$) at a higher temperature ($35^\circ C$.) for a period of 1 hr can therefore be expected to increase the sensitivity of irradiated McCoy cell culture for the isolation of *Chlamydia*.

The authors are grateful to the staff of Moorfields Eye Hospital and other hospitals for referring their patients, to colleagues at the External Eye Diseases Clinic and the Diagnostic Clinic, Moorfields Eye Hospital, for the collection of clinical specimens, to Mrs. Bryony Phillips, B.Sc., of the computer section, Institute of Ophthalmology, for statistical advice, and to Miss Jan Johnstone for secretarial assistance.

We are indebted to the Research and Development Division of the Department of Health and Social Security for a grant in support of this work.

References

- DARUGAR, S., JONES, B. R., KINNISON, J. R., VAUGHAN-JACKSON, J. D., and DUNLOP, E. M. C. (1972) *Brit. J. vener. Dis.*, **48**, 416
- , TREHARNE, J. D., DWYER, R. St. C., KINNISON, J. R., and JONES, B. R. (1971) *Brit. J. Ophthal.*, **55**, 591
- DUNLOP, E. M. C., HARE, M. J., DARUGAR, S., JONES, B. R., and RICE, N. S. C. (1969) *J. infect. Dis.*, **120**, 463
- , VAUGHAN-JACKSON, J. D., and DARUGAR, S. (1972) *Brit. J. vener. Dis.*, **48**, 421
- , —, —, and JONES, B. R. (1972) *Ibid.*, **48**, 425
- GORDON, F. B., DRESSLER, H. R., and QUAN, A. L. (1967) *Amer. J. Ophthal.*, **63**, 1044
- , HARPER, I. A., QUAN, A. L., TREHARNE, J. D., DWYER, R. St. C., and GARLAND, J. A. (1969) *J. infect. Dis.*, **120**, 451
- , MAGRUDER, G. B., QUAN, A. L., and ARM, H. G. (1963) *Proc. Soc. exp. Biol. (N.Y.)*, **112**, 236
- , QUAN, A. L., and TRIMMER, R. W. (1960) *Science*, **31**, 733
- JAWETZ, E., and HANNA, L. (1960) *Proc. Soc. exp. Biol. (N.Y.)*, **105**, 207
- Oriel, J. D., REEVE, P., POWIS, P., MILLER, A., and NICOL, C. S. (1972) *Brit. J. vener. Dis.*, **48**, 429
- RICHMOND, S. J., HILTON, A. L., and CLARKE, S. K. R. (1972) *Ibid.*, **48**, 437
- WEISS, E., and DRESSLER, H. R. (1960) *Proc. Soc. exp. Biol. (N.Y.)*, **103**, 691

Action de la centrifugation à grande vitesse sur la sensibilité des cultures de cellules McCoy irradiées pour l'isolement des *Chlamydia*

La centrifugation à $1.800 G$ à $18^\circ C$ pendant une heure des spécimens recueillis en clinique augmente d'une manière significative la sensibilité de la culture de cellules McCoy irradiées pour l'isolement primaire des *Chlamydia*. En montant la température à $35^\circ C$ pendant la centrifugation, le nombre des inclusions obtenues fut 4,1 fois plus élevée que le nombre des inclusions obtenues après centrifugation à $18^\circ C$. Le nombre des inclusions obtenues après centrifugation des isoléments de laboratoire par une centrifugeuse de paillasse tournant dans une cuve de protection à $2.500 G$ à la température de $35-38^\circ C$ pendant une heure, fut légèrement supérieur au nombre des inclusions obtenues après centrifugation de ces spécimens dans une centrifugeuse réfrigérée tournant à $1.800 G$, à $35^\circ C$ pendant une heure.

Cette observation peut servir de base à l'emploi d'une simple centrifugeuse de paillasse pour l'isolement des *Chlamydia* en routine. Cette technique demeure utile pour les laboratoires de campagne et pour ceux qui ne peuvent pas disposer de centrifugeuse à haute vitesse. La centrifugation à $15.000 G$ à $35^\circ C$ pendant une heure des spécimens recueillis en clinique et ayant un titre bas d'infectivité, augmente d'une manière significative le taux de positivité en culture de cellules McCoy irradiées par comparaison avec la centrifugation à $2.500 G$ à $35^\circ C$ pendant une heure; elle augmente aussi significativement le nombre des inclusions obtenues à partir des spécimens cliniques en comparaison avec la centrifugation soit à 2.500 soit à $10.000 G$ à 35° pendant une heure.

L'emploi d'un dispositif de haute centrifugation ($15.000 G$) à une température plus élevée ($35^\circ C$) pendant une heure peut donc laisser espérer une augmentation de la sensibilité des cultures de cellules McCoy irradiées pour l'isolement des *Chlamydia*.